Appendix 2

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

Satoko YAMAHIRA, et al.

Serial No.: 10/568,671 Art Unit: 1651

Filed: February 17, 2006 Examiner: Irene MARX

For: LACTIC ACID BACTERIA CAPABLE OF STIMULATING MUCOSAL IMMUNITY

SUPPLEMENTAL DECLARATION TO THE DECLARATION UNDER 37 C.F.R. SECTION 1.132, FILED ON MARCH 13, 2009

Honorable Commissioner of Patents and Trademarks

Washington, D.C. 20231

SIR:

I, Yoshito TAJIRI declare that:

1) I am a Japanese citizen, residing at Twin-Harmony A103-gou, 2-1, Ogoto 3-choume, Otsu-shi, Shiga 520-0101, Japan.
2) I graduated from Tokyo University of science, Department of Applied Biological Science, in March 1995. I also graduated from the Graduate School of Tokyo University, and received a Master's Degree in Agriculture in March 1996.
3) I have been employed by OTSUKA PARMACEUTICAL CO., LTD., since April 1996. Since 1996, I have engaged in the research and development of products containing lactic acid bacteria.
4) I am familiar with the subject matter of said application as well as the disclosures in the cited references.
5) In order to demonstrate that *Lactobacillus plantarum* ONRIC b0240

now belongs to *Lactobacillus pentosus*, the following experiments were carried out under my direction and supervision.

Re-identification of *Lactobacillus plantarum* ONRIC b0240

Background

When F. Bringel et al. expounded *L. plantarum* subsp. *argentoratensis* in 2005, they reported that close species of *L. plantarum* can be identified by conducting a molecular phylogeny analysis on the base sequence of the recA gene[1]. Based on this report, we clarified whether *L. plantarum* ONRIC b0240 (hereinunder referred to as b0240) belongs to *L. plantarum* subsp. *plantarum* or *L. plantarum* subsp. *argentoratensis*.

Purpose

To clarify the categorization of *L. plantarum* ONRIC b0240 based on information from the base sequence of the recA gene.

Method

1. Sample strain

L. plantarum ONRIC b0240

2. Culturing method

The sample strain was inoculated in an MRS agar medium and cultured under anaerobic conditions at 37°C for 48 hours.

3. DNA extraction method

The bacteria cultured in the MRS agar medium were collected and extracted using an UltraClean Microbial DNA Kit (Funakoshi: Cat. No. 12224-50). The extracted DNA (genomic DNA) was stored at 4°C until used.

4. PCR reaction

Preparation of reaction mixture :

Genomic DNA	1 µL
Pre-mix cocktail	49 µL

Pre-mix cocktail (40 reactions)	
$_{EX}$Taq (Hot start version)	9 µL
10 x Buffer	200 µL
dNTP mixture	160 µL
Forward primer	36 µL
Reverse primer	36 µL
D.W.	1559 µL

Reaction conditions:
95°C, 5 min.→{95°C, 45 sec.→57°C, 45 sec.→72°C, 90 sec.}x 25 cycles→72°C, 7 min.

5. primer

Partial base sequence of recA (amplification size: approximately 540bp)

- For gene amplification
  - Forward
    - 158F: 5′-GTGGCTACCCACGTGGCCGGA-3′ (21 mer)
  - Reverse
    - 702R: 5′-ACACGGTTACCAATAATATTG-3′ (21 mer)
- For base sequence determination
  - Forward:
    - 163F: 5′-TACCCACGTGGCCGGA-3′ (16 mer)
  - Reverse
    - 702Rs: 5′-TTACCAATAATATTG-3′ (15 mer)

6. Confirmation of target gene amplification

Electrophoresis was conducted for approximately 40 minutes at 100 V in a Mupid2 electrophoresis unit using a 2.0% Nusieve 3:1 agarose gel. The sample was immersed in ethylene bromide for 10 minutes and washed with tap water for approximately 5 minutes. Gene amplification was then measured using a UV illuminator.

7. Purification of PCR product

Primers, nucleotides, enzymes, salts, etc., were removed from the reaction mixture in which amplification was observed using a Rapid PCR Purification System.

8. Base sequence determination

The reaction mixture in which amplification was observed, together with the primers for base sequence determination, was sent to Biomatrix Research, Inc., (270-0101 105 Higashi-Fukai, Nagareyama-city, Chiba phone: 04-7153-8810 facsimile: 04-7153-7820) under refrigeration for base sequence determination.

9. Identification of sample strain

Data of the determined recA partial base sequence and other *L. plantarum* groups were subjected to molecular phylogeny analysis by the Neighbor-Joining method using the molecular phylogeny analysis software CLUSTAL W. A genealogical tree was prepared using the genealogical tree preparation software Tree View. The base sequence information of other *L. plantarum* groups used was as follows: *L. plantarum* subsp. *plantarum* ATCC14917^T (Accession No. AJ621668), *L. plantarum* subsp. *argentoratensis* DK022^T (Accession No. AJ640079), *L. paraplantarum* LMG16673^T (Accession No. AJ621662), *L. pentosus* LMG10755^T (Accession No. AJ621666) L. paracasei subsp. paracasei NCFB151^T (Accession No. AJ621664) (used as out-groups).

By referring to the prepared genealogical tree, the type strain to which the sample strain was closest was determined and identified.

Results

Alignment:

CLUSTAL W (1.83) multiple sequence alignment

```
AJ640079        GCGTTGGGTGTTGGTGGCTACCCACGTGGCCGGATCGTGGAAATCTATGGTCCTGAAAGT
AJ621668        GCGTTGGGTGTCGGCGGCTACCCACGTGGTCGGATCGTGGAAATCTACGGTCCTGAAAGT
AJ621662        GCGTTGGGTGTTGGCGGCTACCCACGTGGCCGGATCGTAGAAATCTATGGTCCTGAAAGT
AJ621666        GCATTAGGCGTTGGTGGTTACCCACGTGGCCGAATCGTTGAAATTTATGGCCCTGAAAGT
ONRIC           GCATTAGGCGTTGGTGGTTACCCACGTGGCCGAATCGTTGAAATTTATGGCCCTGAAAGT
AJ621664        GCACTTGGTGTTGGGAGCTTTCCGCGCGGACGAATTGTCGAAATTTATGGACCGGAAAGT
                ** * ** ** **  * *  ** ** ** ** ** ** ***** ** ** ** ******

AJ640079        TCAGGTAAAACGACCGTAGCACTACATGCGGTTGCTGAAGTTCAAAAGCAGGGTGGCACG
AJ621668        TCAGGTAAAACGACCGTGGCACTACATGCGGTTGCTGAAGTTCAAAAGCAGGGTGGTACG
AJ621662        TCAGGTAAAACGACCGTTGCGTTACACGCGGTGGCTGAAGTTCAAAAGCAAGGTGGCACG
AJ621666        TCCGGTAAAACGACCGTTGCACTACACGCGGTCGCTGAAGTTCAAAAGCAAGGCGGGACG
```

```
ONRIC           TCCGGTAAAACGACCGTTGCACTACACGCGGTCGCTGAAGTTCAAAAGCAAGGCGGGACG
AJ621664        TCCGGCAAAACAACCGTCGCGCTGCATGCTGTTGCTGAAGTGCAGAAGCAAGGCGGTACT
                ** ** ***** ***** **  * ** ** ** ******** ** ***** ** ** **

AJ640079        GCAGCGTATATCGATGCCGAAAATGCACTGGATCCCGTTTACGCGGAACACCTCGGGGTC
AJ621668        GCGGCCTATATCGATGCTGAAAACGCACTAGACCCCGTTTATGCGGAACACCTAGGGGTC
AJ621662        GCAGCTTATATTGATGCTGAAAATGCCTTAGACCCTATTTATGCGGAACATTTGGGGGTT
AJ621666        GCCGCCTATATCGATGCTGAAAACGCCTTGGATCCGGTTTACGCGGAACATTTAGGTGTC
ONRIC           GCCGCCTATATCGATGCTGAAAACGCCTTGGATCCGGTTTACGCGGAACATTTAGGTGTC
AJ621664        GCCGCCTATATTGATGCCGAGAATGCGATGGATCCCAAGTACGCAACTGCTTTGGGCGTC
                ** ** ***** ***** ** ** **  * ** **    ** **        * ** **

AJ640079        AACATCGATGACCTCTTACTCTCACAACCAGACACTGGTGAACAAGGGCTTGAAATTGCA
AJ621668        AACATTGATGACCTGTTACTTTCGCAACCAGATACTGGTGAACAAGGGCTTGAAATTGCA
AJ621662        AATATCGATGATCTATTGCTCTCGCAACCAGATACTGGTGAACAGGGGCTTGAAATCGCA
AJ621666        AACATTGATGATTTGTTACTTTCACAACCAGATACTGGTGAACAAGGTCTTGAAATCGCG
ONRIC           AACATTGATGATTTGTTACTTTCACAACCAGATACTGGTGAACAAGGTCTTGAAATCGCG
AJ621664        AATATTGACGAATTATTGCTCTCCCAGCCAGACACAGGCGAACAAGGGCTGGAGATTGCT
                ** ** ** **  * ** ** ** ** ***** ** ** ***** ** ** ** ** **

AJ640079        GATGCCTTAGTTTCCAGTGGTGCGGTCGACATTTTAGTCGTTGATTCAGTCGCGGCCTTA
AJ621668        GATGCCTTAGTTTCCAGTGGTGCGGTCGATATTTTAGTTGTTGACTCGGTGGCGGCCTTA
AJ621662        GATGCCTTAGTTTCTAGTGGTGCCGTTGATATTTTGGTTGTTGACTCAGTTGCGGCTTTA
AJ621666        GATGCTTTAGTTTCCAGTGGCGCGGTTGATATCTTAGTTGTCGATTCAGTTGCGGCGTTA
ONRIC           GATGCTTTAGTTTCCAGTGGCGCGGTTGATATCTTAGTTGTCGATTCAGTTGCGGCGTTA
AJ621664        GATGAACTAGTCGCGTCTGGTGCCATTGATATTGTGGTGATTGATTCAGTTGCTGCTTTG
                ****   ****  *   *** **  * ** **  * **  * ** ** ** ** ** **

AJ640079        GTGCCACGTGCCGAAATTGAAGGTGAAATGGGTGACGCACACGTTGGGTTACAAGCGCGG
AJ621668        GTGCCACGTGCCGAAATTGAAGGTGAAATGGGTGACGCACACGTTGGGTTACAAGCGCGG
AJ621662        GTTCCACGGGCCGAAATTGAAGGTGAAATGGGTGACGCGCACGTTGGGTTACAAGCTCGA
AJ621666        GTACCACGGGCCGAAATTGAAGGTGAAATGGGTGACGCCCACGTTGGGTTACAAGCCCGG
ONRIC           GTACCACGGGCCGAAATTGAAGGTGAAATGGGTGACGCCCACGTTGGGTTACAAGCCCGG
AJ621664        GTGCCGCGGGCTGAGATTGAGGGCGATATGGGGGATGCCCATGTTGGCTTGCAAGCCCGA
                ** ** ** ** ** ***** ** ** ***** ** ** ** ***** ** ***** **
```

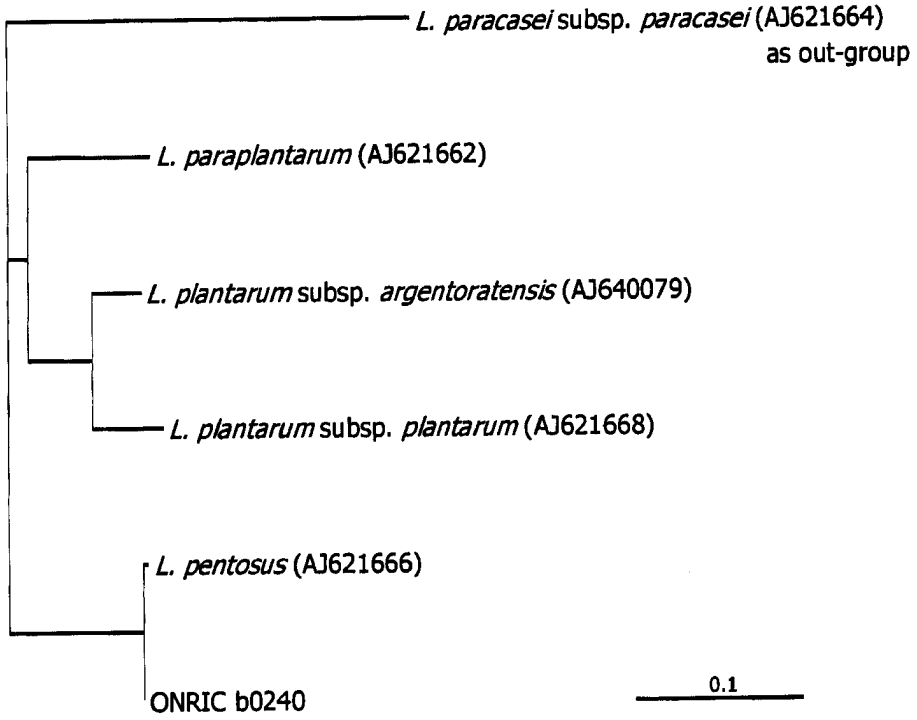
```
AJ640079        TTGATGTCACAAGCCCTTCGGAAGTTATCAGGAACATTGAACAAAACGAAGACCATCGCG
AJ621668        CTGATGTCACAAGCGCTCCGGAAGTTATCAGGGACATTGAACAAAACCAAGACAATCGCG
AJ621662        CTCATGTCACAGGCATTACGAAAACTATCCGGAACGTTGAACAAGACTAAGACGATTGCA
AJ621666        TTAATGTCGCAAGCGTTGCGGAAGTTATCCGGGACTTTGAACAAGACCAAGACCATCGCA
ONRIC           TTAATGTCACAAGCGTTGCGGAAGTTATCCGGGACTTTGAACAAGACAAAGACCATCGCA
AJ621664        TTAATGTCACAGGCGTTACGTAAGTTATCCGGTTCCATCAATAAGACAAAAACGATTGCT
                 * ***** ** **  * ** **  **** **  *  * ** ** ** ** ** ** **

AJ640079        TTATTTATTAATCAAATTCGTGAAAAAGTCGGCGTGATGTTTGGTAATCCCGAAACGACT
AJ621668        TTATTTATCAATCAAATTCGTGAAAAAGTTGGTGTGATGTTTGGTAATCCTGAAACGACT
AJ621662        TTATTTATTAATCAAATTCGTGAAAAAGTCGGGGTGATGTTTGGTAATCCCGAGACGACT
AJ621666        CTATTTATTAACCAAATTCGTGAAAAAGTTGGCGTGATGTTTGGAAACCCTGAAACGACC
ONRIC           CTATTTATTAACCAAATTCGTGAAAAAGTTGGCGTGATGTTTGGAAACCCTGAAACGACC
AJ621664        TTATTCATTAATCAGATTCGTGAGAAAGTCGGGATCATTTTTGGGAGTCCGGAAACGACA
                 **** ** ** ** ******** ***** **  * ** ***** *  ** ** *****

AJ640079        CCTGGTGGTCGGGCCTTGAAGTTCTACGCCACGATTCGTTTGGAAGTTCGGCGGGCAGAA
AJ621668        CCTGGTGGTCGGGCCTTGAAATTTTACGCCACGATTCGTTTGGAAGTACGGCGGGCAGAA
AJ621662        CCGGGTGGTCGGGCGTTGAAATTCTACGCAACAATTCGGTTGGAAGTTCGGCGGGCTGAA
AJ621666        CCTGGTGGTCGGGCTCTGAAGTTCTATGCGACGATTCGACTTGAAGTTCGTCGTGCTGAA
ONRIC           CCTGGTGGTCGGGCTCTGAAGTTCTATGCGACGATTCGACTTGAAGTTCGTCGTGCTGAA
AJ621664        CCAGGTGGTCGTGCATTGAAGTTTTATGCGACTGTTCGGTTAGAAATCCGGCGGTCGGAA
                ** ******** **  **** ** ** ** **  ****  * *** * ** **  * ***

AJ640079        CAGATTAAGGAAGGAACC
AJ621668        CAGATCAAGGAAGGAACC
AJ621662        CAAATTAAAGAGGGAACC
AJ621666        CAAATCAAGGAAGGAACC
ONRIC           CAAATCAAGGAAGGAACC
AJ621664        CAGATCAAGACAGGCGCA
                ** ** **    **  *
```

Phylogenetic tree



Results

It became clear that the strain of the present invention, which had been identified as *L. plantarum* ONRIC b0240, belongs to *L. pentosus*, judging from the results of the molecular phylogeny analysis conducted on the partial recA base sequence. Accordingly, the strain of the present invention is categorized as *L. pentosus* ONRIC b0240 henceforth.

Reference :

1) F. Bringel, et al., Int. J. System. Evol. Microbiol. (2005), 55, 1629–1634

.....

I, undersigned, declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: 2009/10/29

Yoshito TAJIRI